Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 339 (2005) 262–270
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Antibody microarrays for native toxin detection

Victor C. Rucker, Karen L. Havenstrite, Amy E. Herr *

Biosystems Research Department, Sandia National Laboratories, Livermore, CA 94551, USA

Received 18 November 2004

Abstract

We have developed antibody-based microarray techniques for the multiplexed detection of cholera toxin β-subunit, diphtheria toxin, anthrax lethal factor and protective antigen, *Staphylococcus aureus* enterotoxin B, and tetanus toxin C fragment in spiked samples. Two detection schemes were investigated: (i) a direct assay in which fluorescently labeled toxins were captured directly by the antibody array and (ii) a competition assay that employed unlabeled toxins as reporters for the quantification of native toxin in solution. In the direct assay, fluorescence measured at each array element is correlated with labeled toxin concentration to yield baseline binding information (Langmuir isotherms and affinity constants). Extending from the direct assay, the competition assay yields information on the presence, identity, and concentration of toxins. A significant advantage of the competition assay over reported profiling assays is the minimal sample preparation required prior to analysis because the competition assay obviates the need to fluorescently label native proteins in the sample of interest. Sigmoidal calibration curves and detection limits were established for both assay formats. Although the sensitivity of the direct assay is superior to that of the competition assay, detection limits for unmodified toxins in the competition assay are comparable to values reported previously for sandwich-format immunoassays of antibodies arrayed on planar substrates. As a demonstration of the potential of the competition assay for unlabeled toxin detection, we conclude with a straightforward multiplexed assay for the differentiation and identification of both native *S. aureus* enterotoxin B and tetanus toxin C fragment in spiked dilute serum samples.

Keywords: Array; Immunoassay; Anthrax; SEB; Tetanus; Cholera; Toxin; Biosensor

Considerable effort is currently directed toward generating robust devices for toxin and pathogen detection. Arrays of both proteins and nucleic acids are increasingly finding use in this area [1]. Microarrays have features attractive in a detection platform: portability, low rate of false positives, little time for analysis, inexpensive components, and flexibility with regard to analytes that can be studied [2,3]. Microarray-based detectors have the potential for facile application in clinical and field-ready diagnostic devices.

Protein microarrays are a novel technology for quickly detecting and identifying proteins in solution. Related to nucleic acid microarrays, protein microarrays employ surface-immobilized proteins as specific capture reagents for solution-phase proteins [4,5]. Arrayed antibodies are one type of capture reagent employed in the construction of protein microarrays [6]. With antibody or protein microarrays, the ability to use spatial information to identify a unique protein captured from solution depends on the specificity and location of the immobilized capture antibody. Unless the captured antigen is covalently labeled with a fluorophore, most immunochemical techniques employ a second, fluorescently labeled antibody that sandwiches the surface-captured antigen in a highly specific fashion. Owing to their high specificity and affinity, antibody microarrays have become common in clinical diagnostics research where serum (or another bodily fluid) is screened for biomarkers indicative of disease [7,8].

Historically, nucleic acids-based microarrays have been employed successfully both for identifying

* Corresponding author. Fax: +1 925 294 3020.
E-mail address: aeherr@sandia.gov (A.E. Herr).

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.01.030

pathogens based on genetic signatures and for understanding the biological response of an organism after exposure to chemical toxins and mutagens [9,10]. Ligler and co-workers [11–16] have successfully employed arrayed antibodies in a microfluidic format for the fluorescent identification of microorganisms and toxins using a sandwich immunoassay, and ganglioside and G protein-coupled receptor arrays have been constructed for the detection of cholera, tetanus toxin, and botulinum toxin. Enzyme-linked immunosorbent assays (ELISAs)[1] for detecting *Staphylococcus aureus* enterotoxin B (SEB) in cheese, and a recent approach that integrates the orthogonal techniques of nucleic and protein microarrays for differentiating viruses in infection, represent some of the reported benchmarks [17,18]. Non-microarray techniques, such as Biacore surface plasmon resonance, capillary electrophoresis-based immunoshift assays, and advanced imaging techniques, all have been brought to focus on the issue of developing general platforms for the detection of pathogens and toxins [19–22]. Although there are a variety of non-fluorescence techniques for imaging microarrays, the novelty of the approach or the special expertise required appears to have marginalized these non-fluorescence-based approaches from the mainstream [23–26].

We are ultimately interested in antibody microarrays for the detection of illness-causing proteins, referred to here as toxins [27]. In this article, we report on the construction and implementation of a six-element monoclonal antibody microarray for the detection and identification of toxins. Six unique, fluorescently labeled toxins interacting with arrayed antibodies were examined to determine the strength of the antibody–toxin interaction in both buffer and bovine serum diluted 50-fold. Dilute bovine serum spiked with toxin was chosen as a complex biological mixture and, hence, as an indicator of potential interactions between the arrayed antibodies or spiked toxin and irrelevant biomolecules found in the serum. In addition, because the added toxin is the only labeled protein in solution, spiking in the toxin allowed us to analyze the binary antigen–antibody interaction in the absence of potentially confounding cross-reactivity from nonspecific, labeled biomolecules, as would be the case in whole serum-labeling experiments. Both dissociation constants and limits of detection (LODs) for the antibody microarray are reported. The cross-reactivity between labeled toxins and the six immobilized antibodies was also examined to ensure that multiplexed assays would not suffer from nonspecific interactions.

We also report on experiments directed toward the ultimate goal of detecting and identifying unlabeled toxins at low concentration. To this end, a competition assay was designed for the detection and characterization of unlabeled toxins in solution. Competition assays have been reported previously for the detection of toxins using immobilized gangliosides and G protein-coupled receptors, whereas others have used a competition assay for serum-profiling experiments [14–16,28]. In the data reported here, the 50% inhibition constants (IC_{50}) for the competition between fluorescently labeled reporter toxin and unlabeled toxin are characterized for all six analytes in buffer and diluted bovine serum. Both the calculated inhibition constants (K_i) for the binding of the unlabeled toxin to the immobilized antibodies and the calculated LODs using this competition assay for native toxin detection are reported. Finally, as a demonstration of the technique, a multiplexed competition assay that allows the detection and identification of both native SEB and tetanus toxin C fragment (TTC) is presented.

Materials and methods

Antibodies and analytes

TTC was obtained from Roche Applied Science (Indianapolis, IN, USA). SEB, rhodamine-labeled β-subunit of cholera toxin (CT), and diphtheria toxin (DT) were purchased from ListLabs (Campbell, CA, USA). *Bacillus anthracis* lethal factor (LF) and protective antigen (PA) were purchased from EMD Biosciences (San Diego, CA, USA). Molecular weights of the toxins were calculated from data available on PubMed (www.pubmed.net), and the values we employed were 13,956 g/mol for CT β-subunit, 51,970 g/mol for DT, 93,780 g/mol for LF, 76,030 g/mol for PA, 71,768 g/mol for TTC, and 34,090 g/mol for SEB. Unless otherwise stated, all monoclonal antibodies were obtained from Biødesign International (Saco, ME, USA). Monoclonal α-TTC was obtained from Roche Applied Science. α-LF and α-PA were obtained from Advanced Immunochemicals (Long Beach, CA, USA).

Unless otherwise stated, all other reagents were from Sigma–Aldrich (St. Louis, MO, USA). Briefly, antigens were labeled with Alexa Fluor 532 as per the manufacturer's protocol (Molecular Probes, Eugene, OR, USA). Unincorporated fluorophore was removed using Bio-Rad (Hercules, CA, USA) Bio-gel P-6 spin columns as per the manufacturer's instructions. The fluorophore-to-protein ratio was determined by absorption spectroscopy as per the manufacturer's instructions, and for all

[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; SEB, *Staphylococcus aureus* enterotoxin B; LOD, limit of detection; IC_{50}, 50% inhibition constant; TTC, tetanus toxin C fragment; CT, cholera toxin; DT, diphtheria toxin; LF, lethal factor; PA, protective antigen; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PBST, 0.1% (w/v) Tween 20 in phosphate-buffered saline.

proteins this ratio was between 3 and 7 mol fluorophore per mole protein.

Safety consideration

All work was performed at the Chemical and Radiation Detection Laboratory, a biosafety level 2 facility, at Sandia National Laboratories (Livermore, CA, USA). Strict safety and security precautions were exercised in the storage and handling of toxin samples. Safety procedures, as indicated in the appropriate Material Safety Data Sheet forms, should be adhered to at all times. A 20% bleach solution was used to disinfect all equipment, benchtops, and instruments. The disinfectant treatment was followed by a water rinse. Solutions containing toxins, as well as contaminated disposables, were treated with a 20% bleach solution prior to disposal.

Array instrumentation and fabrication

The arrayer used for spotting was constructed in-house from an instrument design based on that of DeRisi et al. [29], and the instrument employs components from Parker Automation Daedal Division (Irwin, PA, USA). Software interface with the arrayer is through a Galil controller card controlled using CamSoft CNC software (Wildeomar, CA, USA). Split pins for spotting were purchased from Majer Precision Engineering (Tempe, AZ, USA).

All antibodies were spotted at 70% relative humidity onto epoxide-functionalized slides (Telechem International, Sunnyvale, CA, USA) at a concentration of 1 mg/ml in a 3:7 glycerol:phosphate-buffered saline (PBS) solution. To ensure mechanical printing precision, one pin was used repetitively, with agitated washing when necessary, to spot all antibodies examined [30,31]. All antibodies were spotted in nine replicates per concentration of antigen examined. Spotted solutions on slides were incubated for 3 h at 70% relative humidity. Prior to the assay, slides were blocked for 1 h with 5% bovine serum albumin (BSA) dissolved in PBST (0.1% (w/v) Tween 20 in PBS) and then rinsed with DI water. A 16-chamber polymer tray was affixed to the slide surface to divide slides into 16 separate wells (Grace Bio-Labs, Bend, OR, USA) spaced 9 mm center to center, thereby allowing us to achieve greater economy per experiment. Antigen dissolved in PBST supplemented with 1 mg/ml BSA was aliquoted into each chamber (50 μl each) and allowed to equilibrate for 1 h. In cases where the interaction was determined in bovine serum, serum was diluted 50-fold directly into PBST supplemented with 1 mg/ml BSA. Incubation times longer than 1 h produced no marked difference in the observed interaction (data not shown). After removing the antigen solution, slides were washed with PBST three times, rinsed in distilled water, and dried under a stream of N_2 gas.

Microarray imaging and data analysis

Slides were imaged on an Axon Instruments (Union City, CA, USA) slide scanner employing a 532-nm diode laser. Typical laser settings were 100% power, and typical photomultiplier tube (PMT) gain settings were between 700 and 1000. All image quantification was performed using GenePix software provided with the scanner. Functions internal to the software determine and correct for background fluorescence signal by subtracting the local median background fluorescence from the average fluorescence intensity at each array element. The background corrected and averaged fluorescence signals, referred to here and throughout the article as apparent occupancy (θ_{app}), were normalized and plotted against the concentration ($[C]_0$) of added protein. Normalized occupancy data ($[C]_0$, θ_{app}) were fit to the equation $\theta_{fit} = K_a[C]_0/(1 + K_a[C]_0)$ for a Langmuir binding isotherm [32]. A least-squares fit was used to determine the association constant, K_a. All fits had correlation coefficients $\geqslant 0.97$.

Using the fit of the data to the Langmuir equation, a stringent metric of 3 standard deviations (3σ) above the baseline signal (where $\theta_{fit} = 0$) in the direct assay or 3σ below the maximum signal (where $\theta_{fit} = 1$) in the competition assay was used to calculate LODs [6–8]. The concentration where $\theta_{fit} = 0 + 3\sigma$ or $\theta_{fit} = 1 - 3\sigma$ can be solved by rearranging the Langmuir equation to $[C]_0 = \theta_{fit} K_d/(1 - \theta_{fit})$ for the direct assay or to $[C]_0 = \theta_{fit} IC_{50}/(1 - \theta_{fit})$ for the competitive assay.

In the competition assay, where unlabeled toxin competes with labeled toxin for binding to the immobilized antibodies, inhibition constants were calculated from 50% inhibition constant values by the equation $K_i = IC_{50}/(1 + [\text{reporter}]/K_d)$, where [reporter] refers to the fixed concentration of fluorescent toxin and K_d is the dissociation constant determined from the direct assay [33]. The level of agreement between K_d (measured in the direct immunoassay) and K_i (measured in the competition immunoassay) indicates the effect, if any, that fluorescently labeling the antigen has on the antigen–antibody recognition event.

Results and discussion

Direct assay for detection of toxins

The affinity of antibodies spotted onto epoxide surface-functionalized glass slides for fluorescently labeled antigen was evaluated in both buffer and diluted bovine serum solutions. Fig. 1A shows the format for the direct assay, with representative data exhibited in Fig. 1B. Immobilized antibodies were spotted in segregated arrays that were divided into 16 groups on one slide using a multiwell polymer divider to isolate each group. Each

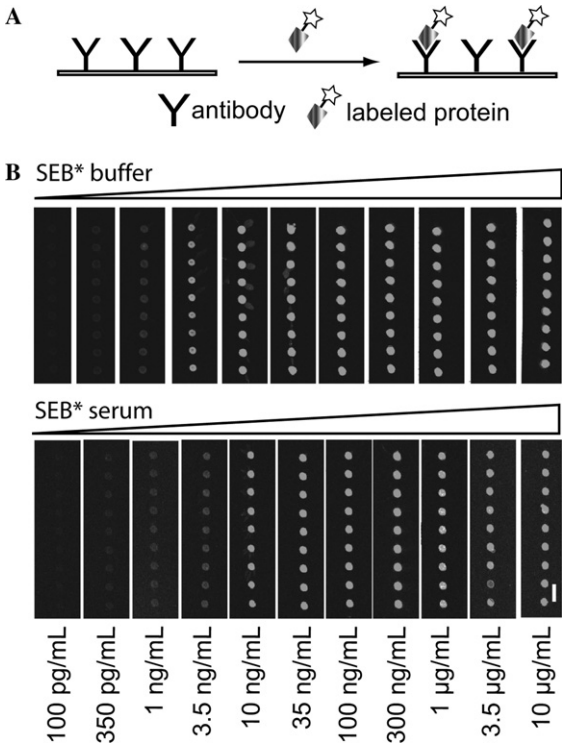

Fig. 1. (A) Direct assay format. Immobilized monoclonal antibodies are exposed to a concentration gradient of fluorescently labeled toxin in both buffer and diluted bovine serum. (B) Representative images for the direct detection of fluorescently labeled SEB (SEB*) over a concentration range of 10 µg/ml–100 pg/ml detected in both PBST containing 1 mg/ml BSA or bovine serum diluted 50-fold into PBST containing 1 mg/ml BSA. The white scale bar at the lower right represents 300 µm. Concentrations of applied, fluorescently labeled SEB for both arrays are indicated below the image.

group was individually exposed to antigen solutions of different concentrations. To generate the images shown in Fig. 1B, 10 µg/ml–100 pg/ml of fluorescently labeled SEB dissolved in either PBST or diluted serum was incubated with the immobilized antibodies. In all cases, concentrations of applied toxin were chosen such that saturation of immobilized antibodies at high concentrations and undersaturation of immobilized antibodies at low concentrations of applied toxin were observed.

A microarray of six immobilized antibodies against the six toxins was examined in buffer and diluted serum to characterize potential cross-reactivity for the interaction between the sextuplet of antibodies and most of the individual labeled antigens. For each applied toxin, cross-reactivity analysis consisted of quantifying and averaging the signals at each unique row of immobilized antibodies and expressing that averaged signal $\bar{n}$, divided by the sum of the averaged signals determined at each of the six individual antibody rows ($\{\bar{n}/[\sum \bar{n}_i/6]\}$, $i = 1$–6), as a measure of specificity (Supplementary Fig. 1). Maximum signal was consistently observed for the specific interaction between antibody and cognate antigen, with low signal being observed for the five expected nonspecific interactions. Often the cross-reactivity between the immobilized antibodies and the applied toxin was so low that no meaningful signal was detected. An exception to the overall low cross-reactivity of our antibody panel was the observed interaction between LF* and PA* (where the asterisk indicates that the antigen is fluorescently labeled) with α-SEB and α-CT. These minimally nonspecific interactions individually account for only approximately 10% of the total summed signal, and the nonspecific interactions may be an artifact of the direct labeling procedure [34].

The data collected for all six direct assays, in both buffer and diluted bovine serum, were normalized and plotted as the apparent occupancy (θ_{app}) of the immobilized antibodies against the applied concentration of fluorescent antigen, as shown in Fig. 2. Minimizing the difference between θ_{app} and θ_{fit} to the Langmuir binding isotherm ($\theta_{fit} = K_a[C]_0/(1 + K_a[C]_0)$) yields an association constant at an applied toxin concentration $[C]_0$ where $\theta_{fit} = 0.5$. The dissociation constants ($K_d = K_a^{-1}$) for the antigen–antibody interaction in both dilute bovine serum and buffer are presented in Table 1, and these values are acceptable for antigen–antibody binding events [35]. Furthermore, it can be seen from the ratio of dissociation constants (K_d^{serum}/K_d^{buffer}) that there is little effect from the complex biological matrix on the performance of the antibody microarray. It is noteworthy that within the population of toxins reported herein, there is a maximum observed discrepancy of 5 for the ratio K_d^{serum}/K_d^{buffer} determined for the interaction of both LF* and CT* with the immobilized antibodies under the two different buffered conditions. Nonspecific interactions between analytes and matrix components in complex solutions have been reported previously to cause deviations in apparent analyte concentrations, suggesting that the variation reported here may result from a similar phenomenon [11].

To evaluate the sensitivity of the antibody microarray for the direct detection of fluorescently labeled antigen, LODs (ranging from a low of 14 ng/ml for the detection of three analytes to a high of 704 ng/ml for the detection of LF* in dilute bovine serum) were calculated as 3σ over the minimum occupancy in the fit of the data to the Langmuir equation and are reported in Table 1. Because we determine the LOD from the standard deviation for the fit of our data, the calculated LODs (from the rearranged Langmuir equation $[C]_0 = \theta_{fit} K_d/1 - \theta_{fit}$) are a convolution of both the noise in the fit and the actual performance of our detector in both buffer and dilute bovine serum. Previously reported LODs for toxins immunochemically detected range from 1.6 ng/ml for CT using a two-antibody sandwich assay to 1000 ng/ml for CT using a ganglioside–antibody sandwich, and the values reported in Table 1 are within this range [11,14]. Importantly, because our experiments in

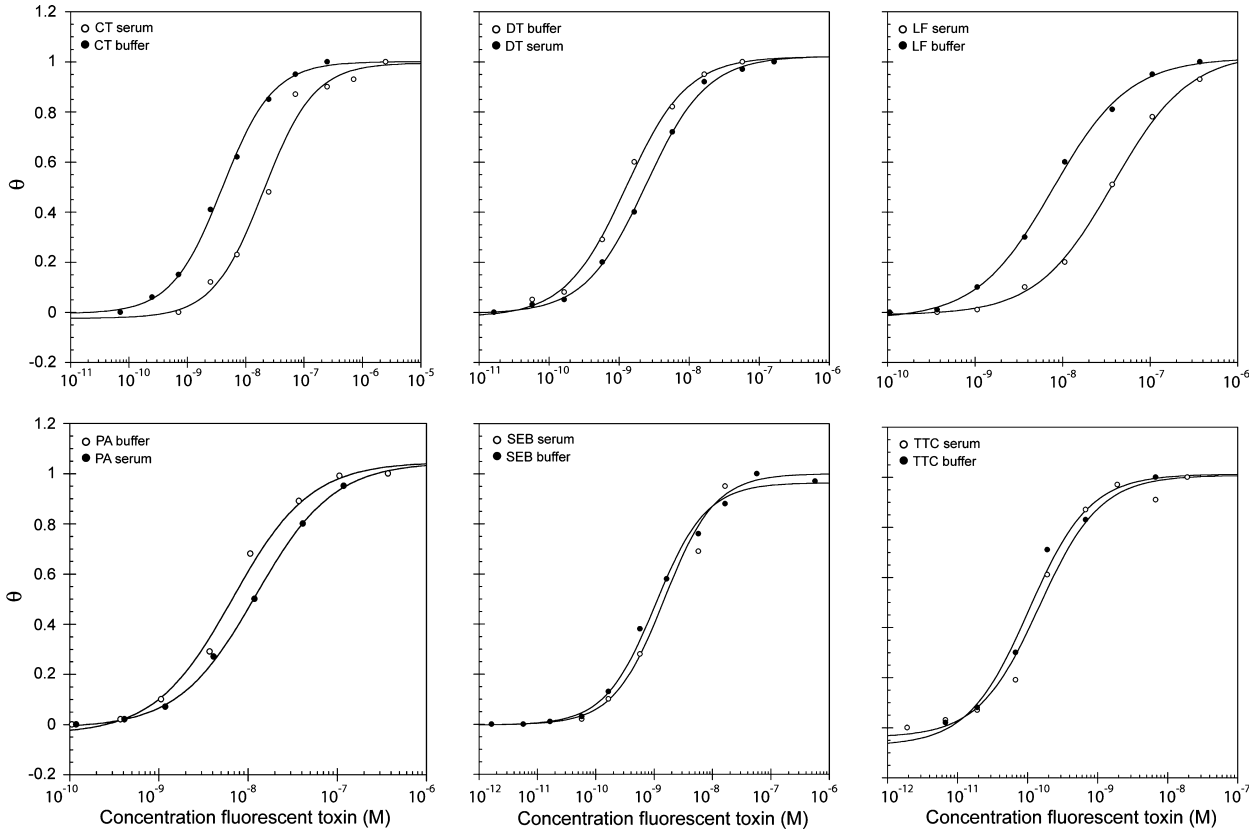

Fig. 2. Normalized data for six fluroescently labeled toxins directly detected in both buffer and diluted bovine serum. Experimentally determined apparent occupancy data (θ_{app}) were plotted on a linear-log scale against the concentration of fluorescent toxin (M) and were fit to the Langmuir binding isotherm, $\theta_{fit} = K_a[C]_0/(1 + K_a[C]_0)$, by minimizing the difference between θ_{app} and θ_{fit}. θ is the immobilized antibody occupancy, K_a is the association constant (units of M^{-1}), and $[C]_0$ is the concentration of added protein. All 12 fits had correlation coefficients $\geqslant 0.97$.

Table 1
Dissociation constants ± standard deviations determined for the direct binding assay between fluorescently labeled toxin and immobilized antibodies in both PBST + 1 mg/ml BSA and bovine serum diluted 50-fold into PBST + 1 mg/ml BSA

Toxin	K_d PBST ($M \times 10^9$)	LOD (ng/ml)	K_d serum ($M \times 10^9$)	LOD (ng/ml)	K_d^{serum}/K_d^{PBST}
CT*	4.0 ± 0.3	13.9	20.0 ± 4.9	52	5.0
DT*	1.3 ± 0.2	52	2.4 ± 0.15	29	2.0
LF*	7.7 ± 0.6	169	38.0 ± 2.5	704	5.0
PA*	6.7 ± 1.0	414	12.0 ± 0.06	14	1.9
SEB*	1.1 ± 0.1	14	1.5 ± 0.3	77	1.4
TTC*	0.15 ± 0.03	19.6	0.15 ± 0.04	56	1.0

diluted bovine serum are intended only to provide characterization of the specific interaction between labeled toxin in solution and immobilized antibody challenged to select the labeled toxin from a complex mixture of biomolecules, we cannot yet comment on the performance of our assay if whole, toxin-contaminated serum samples were labeled with an amine-reactive fluorophore, as is common in many serum-profiling experiments [34].

Competitive assay for detection of native toxins

There are potential drawbacks associated with directly modifying antigens with fluorophore, as is often done in serum profiling, not the least of these being intersampling labeling variation, potentially compromised specificity and/or affinity, and the inability to detect native toxins [34]. To move away from these potential issues, a competition assay was investigated as a basis for toxin detection (Fig. 3A). In the competition assay, three components are involved: the immobilized antibody, the fluorescent toxin (reporter), and the unlabeled toxin of interest. A mixture of the fluorescently labeled reporter, held at a constant concentration, and a concentration gradient of identical, but unlabeled, toxin are applied to the microarray. Because of the competition between the labeled toxin and the unlabeled analyte to be detected, the concentration of reporter

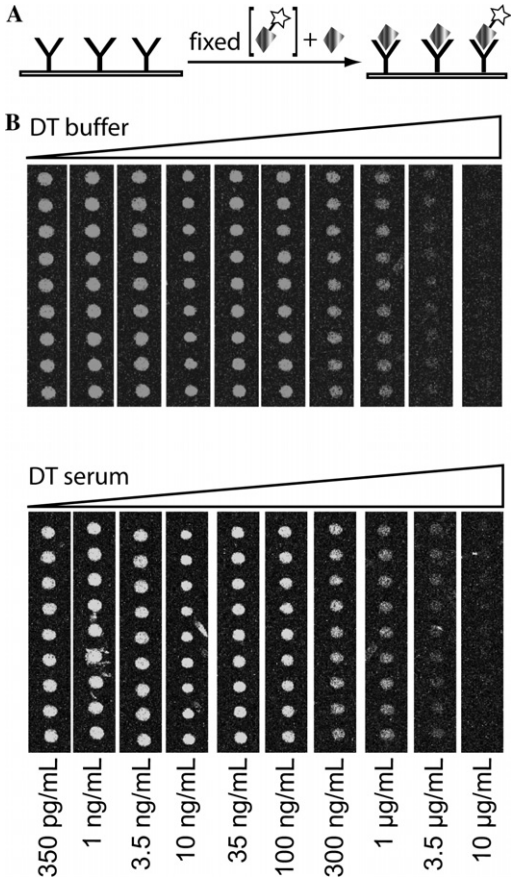

Fig. 3. (A) Competition assay format. A fixed concentration of reporter toxin competes to bind immobilized antibody as the concentration of unlabeled toxin is increased. (B) Representative image for the competition of 19 nM fluorescent DT (reporter) against an added concentration gradient of unlabeled DT ranging from 350 pg/ml to 10 µg/ml. The interaction was examined in both PBST supplemented with 1 mg/ml BSA and bovine serum diluted 50-fold into PBST with 1 mg/ml BSA. Applied concentrations of unlabeled toxin competitor are indicated below the image.

toxin must be as low as possible without compromising signal quality. We chose reporter concentrations that were minimally greater than the LOD determined in the direct assay (Tables 1 and 2). Experimentally, maximum fluorescent signal is observed when the concentration of unlabeled protein (titrant) is zero (Fig. 3B, with DT used as an example), and the fluorescence signal decreases sigmoidally, in this example, toward the minimum signal, $\theta_{fit} \sim 0$, as the concentration of unlabeled toxin is increased from 350 pg/ml to 10 µg/ml.

The competition assay was performed with all six antigens in both buffer and dilute bovine serum with reporter concentrations nominally greater than the LOD determined in the direct assay (Tables 1 and 2). As with the direct assay, the data were normalized to generate θ_{app} values that were plotted against the total concentration of unlabeled protein in solution (Fig. 4). The raw data were fit to the Langmuir equation. In this competition assay, the inflection point of the plot (where $\theta_{fit} = 0.5$) relates to the 50% inhibition constant, that is, the point at which only 50% of the fluorescent reporter is bound by the surface-immobilized antibodies in the presence of unlabeled inhibitor. The IC_{50} value is dependent on the concentration of fluorescent reporter, but with the previously determined K_d values (Table 1), one can readily calculate the concentration of reporter-independent inhibition constant using the equation $K_i = IC_{50}/(1 + [\text{reporter}]/K_d)$ [33]. The dissociation constants in Table 1 and the inhibition constants in Table 2 agree well within an order of magnitude, with a maximum disagreement of a factor of 4.2 noted for the interaction of SEB in buffer. The close agreement between the dissociation constants measured in the direct assay and the inhibition constants calculated from the competition assay implies that covalent modification of the toxin with the fluorophore does not significantly alter the toxin's structure or interfere with the binding between the immobilized antibodies and the labeled antigen in solution.

The competition assay also yields LODs, but unlike in the direct assay where the LOD was defined as the statistically significant signal above the baseline (where θ_{fit} approaches 0), an LOD calculated from a competition assay relates to a reduction of θ_{fit} by 3σ from unity as the concentration of unlabeled competing toxin is increased. The LODs reported in Table 2 for the competition assay are again well within the range of values encountered in the literature for the detection of native toxins by the sandwich assay. Nevertheless, for some cases (e.g., LF in dilute serum, PA in buffer), the LODs are quite high. These apparently high LODs result from two convolved effects: the actual concentration-

Table 2
Inhibition constants ± standard deviations (IC_{50}), calculated inhibition constants (K_i) and LODs for the detection of unlabeled toxin

Toxin	IC_{50} PBST ($M \times 10^9$)	LOD (ng/ml)	K_i PBST ($M \times 10^9$)	IC_{50} serum ($M \times 10^9$)	LOD (ng/ml)	K_i serum ($M \times 10^9$)	[reporter] ($M \times 10^9$)
CT	91 ± 0.6	26	4.7	175 ± 20.0	1200	38.0	72
DT	62 ± 1.0	306	4.0	12 ± 0.15	23.6	1.3	19
LF	13 ± 1.9	1030	7.9	100 ± 14.0	5300	88.4	5
PA	19 ± 3.0	1300	14.6	32 ± 1.3	332	27.4	2
SEB	7.1 ± 1.5	412	4.6	5.6 ± 0.4	52	4.0	0.59
TTC	0.3 ± 0.04	16.8	0.19	0.83 ± 0.1	40.9	0.5	0.097

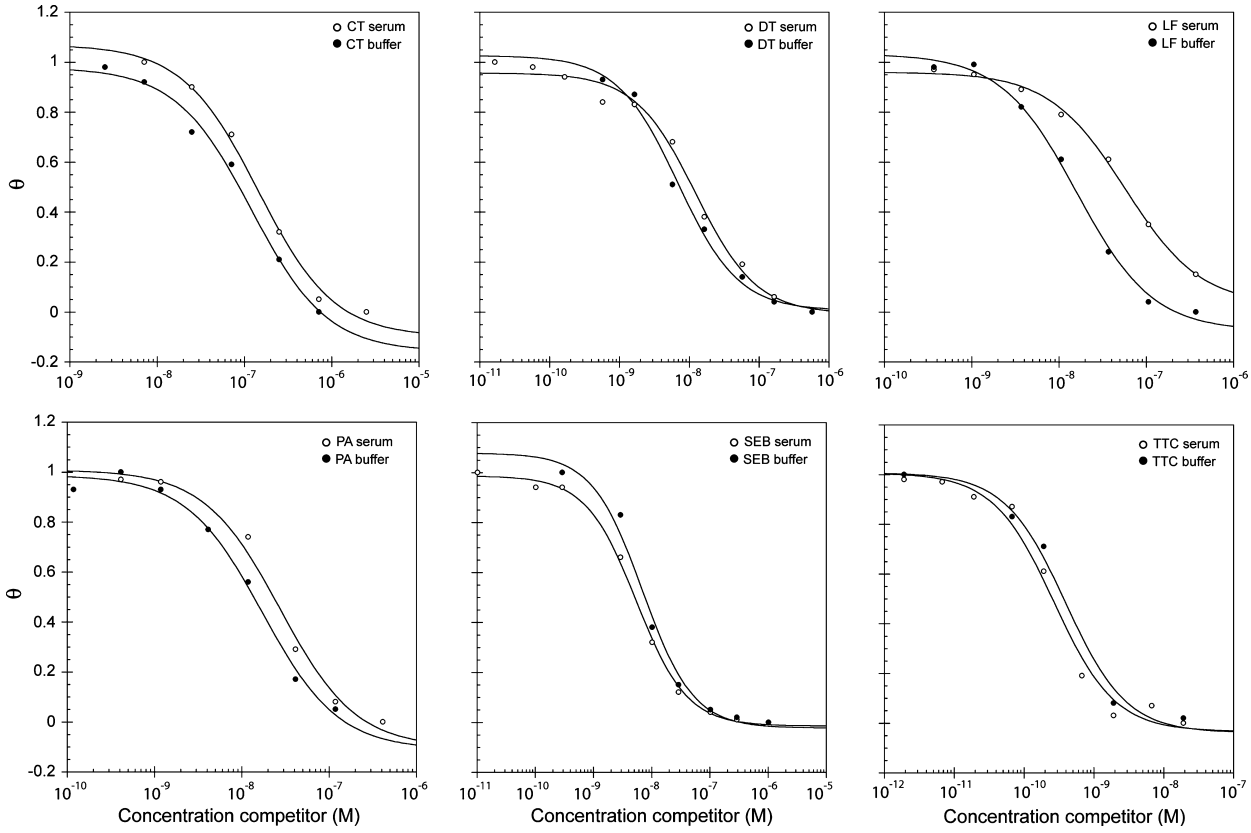

Fig. 4. Normalized competition data for six toxins in both buffer and diluted bovine serum. Experimentally determined apparent occupancy data (θ_{app}) were plotted on a linear-log scale against the concentration of unlabeled toxin (M) and were fit to the Langmuir binding isotherm, $\theta_{fit} = IC_{50}^{-1}[C]_0/(1 + IC_{50}^{-1}[C]_0)$, by minimizing the difference between θ_{app} and θ_{fit}. θ is the immobilized antibody occupancy, IC_{50} is the 50% inhibition constant (units of M), and $[C]_0$ is the concentration of added protein. All 12 fits had correlation coefficients $\geqslant 0.97$.

dependent competition between the unlabeled and labeled toxins for binding to the arrayed antibodies (the higher the reporter concentration, the higher the apparent LOD) and the higher σ in the fits of these data. For the poorest LODs calculated (e.g., PA in buffer), the data never become fully asymptotic to the concentration axis, even at the highest concentration of applied competitor (35 μg/ml) where $\theta_{fit} \sim 0$. This leads to uncertainty in the determination of the lower bound of the isotherm; hence, there is a higher standard deviation for the interpolation of the IC_{50} between the bounds $1 \geqslant \theta_{fit} \geqslant 0$.

Model application: Detection of native SEB and TTC

In most clinical and forensic situations involving unknowns, there is a list of likely toxins or proteins that will be encountered [27]. Laboratory-based ELISA and "dipstick" immunoassays have long been tailored around this fact, and antibody microarrays constructed for unlabeled toxin detection would also be tailored for the detection of a population of suspect, commonly encountered agents. To use the competition assay outlined here in such a format (Fig. 5A), two arrays would need to be compared: a control array to which only the reporter is applied and a test array to which the unknown sample, spiked with a concentration of reporter equal to that added to the control, is applied. By simple comparison of the control and the test array, a reduction in signal would be noted at an antibody element when an unlabeled target toxin is present in solution (panel on left in Fig. 5A). As an illustration of this approach, we constructed a two-plex antibody microarray for the detection of native SEB and native TTC in diluted bovine serum (Fig. 5B). The signal observed at the SEB element in the presence of 29 nM unlabeled SEB (measured fluorescence signal of 1272 ± 1322) is approximately 10% of the original signal ($12{,}623 \pm 170$) observed for the control in the absence of competitor, and the signal observed at the TTC element in the presence of 1.9 nM TTC (1354 ± 150) is approximately 17% of the signal observed for the control in the absence of competitor (7843 ± 594). In both cases, at the concentrations of reporter and unknown used, 90% reduction in intensity for the SEB signal and 83% reduction in intensity for the TTC signal are expected from analysis of the isotherms presented previously in Fig. 4. These results demonstrate the potential of this technique for both

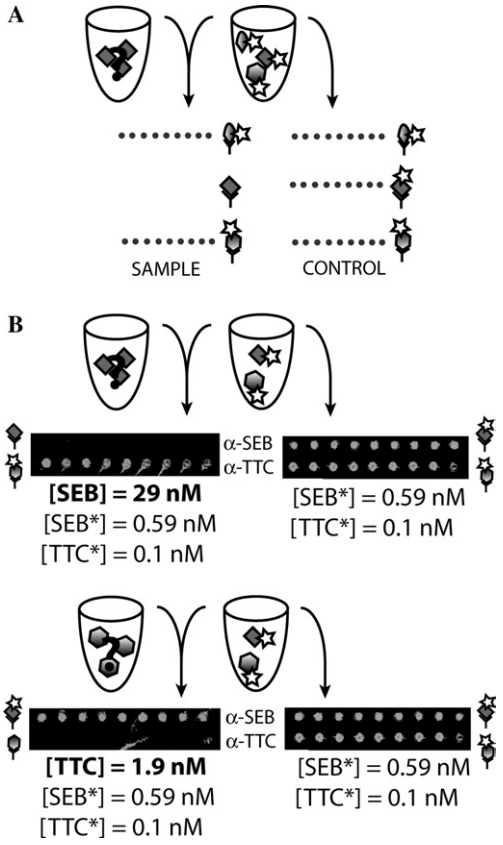

Fig. 5. (A) Format for binary antibody microarray to determine the presence or absence of a toxin. Two arrays are necessary for comparison: a control array of labeled toxins (right) to determine the fluorescence intensity in the absence of unlabeled competitor and an array (left) to which is added the unknown solution spiked with reporter toxin (indicated by star) at the same concentration as applied to the control. (B) SEB signal from 0.59 nM SEB reporter is reduced approximately 90% in the presence of 29 nM native SEB (left). The TTC signal from 0.1 nM TTC is reduced approximately 83% in the presence of 1.9 nM unlabeled TTC (bottom left). In both cases, these reductions are expected from the inhibition curves presented in Fig. 4.

identifying and providing concentration data about unlabeled toxins in biologically relevant sample solutions.

Conclusions

We have reported on two different antibody-based microarray assays for the detection of CT β-subunit, DT, anthrax LF and PA, SEB, and TTC both in buffer and, as a test for detector performance in solutions of complex composition, in diluted bovine serum. To our knowledge, this is the first platform for detecting these toxins using antibody microarrays contact printed onto epoxide-functionalized glass slides. The format chosen for our assay employs fluorescently modified toxins as both a model case for the detection of fluorescently labeled toxins in solution and also as reporters in a competition assay designed to detect unlabeled toxins in a complex sample. The competition assay, involving both labeled and unlabeled toxins, allows the detection of toxins in their native state in both buffer and diluted serum. The microarray format also provides thermodynamic characterization of the arrayed antibody–toxin interactions. Dissociation constants for the direct assay and IC_{50} values for the competition assay were extracted from fitting data generated using the antibody microarray to the Langmuir equation. The LODs for the competition assay to detect unlabeled toxin are, overall, comparable to those achievable using the sandwich immunoassay, but for some toxins our threshold sensitivity is marginally poorer than would be expected from a sandwich assay. There are two primary ways in which to improve the sensitivity of the competition assay: (i) decreasing the standard deviation in the fit of the data to the Langmuir equation by generating isotherms that become fully asymptotic to the concentration axis at high and low concentrations of added competitor and (ii) decreasing the concentration of added reporter such that appreciable reduction in reporter signal occurs at a lower concentration of native toxin. In a final examination of our approach, we designed a multiplexed assay for the discrimination and detection of both native SEB and TTC and demonstrated that this binary assay provides a facile quantitative route to analyzing solutions for the presence or absence of toxins. The choice of toxins examined spans both clinical and forensic interests, further underscoring the general applicability of protein microarrays for detecting and identifying toxins.

Acknowledgments

Sandia is a multiple-program laboratory operated by Sandia Corporation, a Lockheed Martin company, for the U.S. Department of Energy under contract DE-AC04 94AL85000. The authors acknowledge J.C. Brennan for construction of the arrayer and K.W. Hukari for assistance with the CamSoft interface.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2005.01.030.

References

[1] S.S. Iqbal, M.W. Mayo, J.G. Bruno, B.V. Bronk, C.A. Batt, J.P. Chambers, A review of molecular recognition technologies for the detection of biological threat agents, Biosens. Bioelectron. 15 (2000) 549–578.

[2] T. O'Brien, L.H. Johnson III, J.L. Aldrich, S.G. Allen, L.-T. Liang, A.L. Plummer, S.J. Krak, A.A. Boiarski, The development of immunoassays to four biological threat agents in a bidiffractive grating biosensor, Biosens. Bioelectron. 14 (2000) 815–828.
[3] C.A. Rowe, L.M. Tender, M.J. Feldstein, J.P. Golden, S.B. Scruggs, B.D. MacCraith, J.J. Cras, F.S. Ligler, Array biosensor for simultaneous identification of bacterial, viral, and protein analytes, Anal. Chem. 71 (1999) 3846–3852.
[4] H. Zhu, M. Snyder, Protein chip technology, Curr. Opin. Chem. Biol. 7 (2003) 55–63.
[5] G. MacBeath, S.L. Schreiber, Printing proteins as microarrays for high-throughput function determination, Science 289 (2000) 1760–1763.
[6] J. Glökler, P. Angenendt, Protein and antibody microarray technology, J. Chromatogr. B 797 (2003) 229–240.
[7] J.C. Miller, H.P. Zhou, J. Kwekel, R. Cavallo, J. Burke, E.B. Butler, B.S. Teh, B.B. Haab, Antibody microarray profiling of human prostate cancer sera: Antibody screening and identification of potential biomarkers, Proteomics 3 (2003) 56–63.
[8] Y. Li, W.M. Reichert, Adapting cDNA microarray format to cytokine detection protein arrays, Langmuir 19 (2003) 1557–1566.
[9] M. Schena, Microarrays as toxin sensors, Pharmacogenom. J. 3 (2003) 125–127.
[10] W.J. Wilson, C.L. Strout, T.Z. DeSantis, J.L. Stilwell, A.V. Carrano, G.L. Andersen, Sequence-specific identification of 18 pathogenic microorganisms using microarray technology, Mol. Cell. Probes 16 (2002) 119–127.
[11] F.S. Ligler, C.R. Taitt, L.C. Shriver-Lake, K.E. Sapsford, Y. Shubin, J.P. Golden, Array biosensor for detection of toxins, Anal. Bioanal. Chem. 377 (2003) 469–477.
[12] C.R. Taitt, G.P. Anderson, B.M. Lingerfelt, M.J. Feldstein, F.S. Ligler, Nine-analyte detection using an array-based biosensor, Anal. Chem. 74 (2002) 6114–6120.
[13] J.B. Delehanty, F.S. Ligler, A microarray immunoassay for simultaneous detection of proteins and bacteria, Anal. Chem. 74 (2002) 5681–5687.
[14] C.A. Rowe-Taitt, J.J. Cras, C.H. Patterson, J.P. Golden, F.S. Ligler, A ganglioside-based assay for cholera toxin using an array biosensor, Anal. Biochem. 281 (2000) 123–133.
[15] Y. Fang, A.G. Frutos, J. Lahiri, Ganglioside microarrays for toxin detection, Langmuir 19 (2003) 1500–1505.
[16] Y. Fang, A.G. Frutos, B. Webb, Y. Hong, A. Ferrie, F. Lai, J. Lahiri, Membrane biochips, BioTechniques 33 (2002) 62–65.
[17] C. Morissette, J. Goulet, G. Lamoureux, Rapid and sensitive sandwich enzyme-linked immunosorbent assay for detection of *Staphylococcal* enterotoxin-B in cheese, Appl. Environ. Microbiol. 57 (1991) 836–842.
[18] A. Perrin, D. Duracher, M. Perret, P. Cleuziat, B. Mandrand, A combined oligonucleotide and protein microarray for the codetection of nucleic acids and antibodies associated with human immunodeficiency virus, hepatitis B virus, and hepatitis C virus infections, Anal. Biochem. 322 (2003) 148–155.
[19] A.N. Naimushin, S.D. Soelberg, D.K. Nguyen, L. Dunlap, D. Bartholomew, J. Elkind, J. Melendez, C.E. Furlong, Detection of *Staphylococcus aureus* enterotoxin B at femtomolar levels with a miniature integrated two-channel surface plasmon resonance (SPR) sensor, Biosens. Bioelectron. 17 (2002) 573–584.
[20] M.T. Lam, C.A. Boulet, X.C. Le, Development of a tetramethylrhodamine-labeled probe for a capillary electrophoresis-based competitive immunoassay of *Staphylococcal* enterotoxin B, Anal. Chim. Acta 457 (2002) 21–28.
[21] E.R. Goldman, A.R. Clapp, G.P. Anderson, H.T. Uyeda, J.M. Mauro, I.L. Medintz, H. Mattoussi, Multiplexed toxin analysis using four colors of quantum dot fluororeagents, Anal. Chem. 76 (2004) 684–688.
[22] Y.M. Bae, B.-K. Oh, W. Lee, W.H. Lee, J.-W. Choi, Immunosensor for detection of *Yersinia enterocolitica* based on imaging ellipsometry, Anal. Chem. 76 (2004) 1799–1803.
[23] M.A. Cooper, Label-free screening of bio-molecular interactions, Anal. Bioanal. Chem. 377 (2003) 834–842.
[24] H-M. Striebel, P. Schellenberg, P. Grigaravicius, K.O. Greulich, Readout of protein microarrays using intrinsic time resolved UV fluorescence for label-free detection, Proteomics 4 (2004) 1703–1711.
[25] R. McKendry, J. Zhang, Y. Arntz, T. Strunz, M. Hegner, H.P. Lang, M.K. Baller, U. Certa, E. Meyer, H.-J. Güntherodt, C. Gerber, Multiple label-free biodetection and quantitative DNA-binding assays on a nanomechanical cantilever array, Proc. Natl. Acad. Sci. USA 99 (2002) 9783–9788.
[26] F. Caruso, E. Rodda, D.N. Furlong, K. Niikura, Y. Okahata, Quartz crystal microbalance study of DNA immobilization and hybridization for nucleic acid sensor development, Anal. Chem. 69 (1997) 2043–2049.
[27] J.M. Madsen, Toxins as weapons of mass destruction: A comparison and contrast with biological-warfare and chemical-warfare agents, Clin. Lab. Med. 21 (2001) 593–605.
[28] R. Barry, T. Diggle, J. Terrett, M. Soloviev, Competitive assay formats for high-throughput affinity arrays, J. Biomol. Screen. 8 (2003) 257–263.
[29] J.L. DeRisi, V.R. Iyer, P.O. Brown, Exploring the metabolic and genetic control of gene expression on a genomic scale, Science 278 (1997) 680–686.
[30] D.S. Rickman, C.J. Herbert, L.P. Aggerbeck, Optimizing spotting solutions for increased reproducibility of cDNA microarrays, Nucleic Acids Res. 31 (2003) e109.
[31] F. Diehl, S. Grahlmann, M. Beier, J.D. Hoheisel, Manufacturing DNA microarrays of high spot homogeneity and reduced background signal, Nucleic Acids Res. 29 (2001) e38.
[32] A. Ben-Naim, Cooperativity and Regulation in Biochemical Processes, Kluwer Academic/Plenum, New York, 2001.
[33] Y.-C. Cheng, W.H. Prusoff, Relationship between inhibition constant (K_i) and concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic-reaction, Biochem. Pharmacol. 22 (1973) 3099–3108.
[34] V.N. Morozov, Protein microarrays: Principles and limitations, in: M. Schena (Ed.), Protein Microarrays, Jones & Bartlett, Sudbury, MA, 2005.
[35] D. Voet, J.G. Voet, C.W. Pratt, Fundamentals of Biochemistry, Wiley, New York, 2002.